

A SIMPLE AND CONVENIENT INOCULATION METHOD USING SMALL WOODEN CHIPS MADE OF DISPOSABLE CHOPSTICKS FOR *ROSELLINIA NECATRIX*, THE CAUSAL FUNGUS OF WHITE ROOT ROT DISEASE ON FRUIT TREES

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ABSTRACT

A simple and convenient inoculating method for *Rosellinia necatrix*, the causal fungus of white root rot disease of fruit tree, was developed. The *R. necatrix* W563 was incubated on potato dextrose agar medium (PDA) plate for 1 - 2 weeks at 25°C. The wooden chips (ca. 30 x 5 x 5mm) made of disposable chopsticks were autoclaved at 120°C for 20 minutes and placed on the colony of W563 and incubated for 1 week at 25°C. Each infected wooden chip was cut off into uniform length of 20mm, and was attached onto the stem base of one year old of Chinese crab apple (*Malus prunifolia*) seedling grown in the small plastic pot and tightly fixed with a Parafilm tape. The inoculated region was covered with soil and the seedling was incubated in the air-conditioned greenhouse at 25°C. Three weeks after inoculation, four of five seedlings inoculated with W563 showed decline in newly developed twigs followed by wilt and fall of the leaves resulting in total death by 6 weeks after inoculation. In another trial, the small wooden chips (ca. 10 x 5 x 5mm) placed and incubated on W563 colony for 2 weeks were perpendicularly inserted into the soil nearby the stem base of the seedlings of yellow lupine (*Lupinus luteus*), apple (*Malus x domestica*) and Japanese pear (*Pyrus pyrifolia*). All the plants inoculated through the insertion of wooden chips showed almost the same symptoms as those of the first experiment, by 6 weeks after inoculation. The above mentioned inoculation methods, especially by means of infested wooden chip insertion, were very stable in their pathogenic effect, simpler and more convenient than the traditional way using the infested soil prepared by mixing with infested wheat bran.

Key words: inoculum, *Malus prunifolia*, *Pyrus pyrifolia*, soil bore disease, *Waribashi*

INTRODUCTION

Rosellinia necatrix is the causal fungus of white root rot disease of many fruit trees and can infect about 170 species, 63 genera and 30 families of plants (Horie et al., 2001; Perez-Jimenez, 2006; Szejnbrtg and Madar, 1980). The infected plant's growth is stunted resulting in weak development of twigs and leaves, and poor fruit production followed by total death regardless of the difference in cultivars. The disease was named after the peculiar symptom that contained bald bundles of white thread-like structures consisting of the pathogen fungus hyphae covering the surface of the stem bases and main roots. In spite of

its severe damage on the commercial fruit production, there is almost no practical effective method to control the disease. As many orchards were developed along slopes in Japan, the protection against white root rot disease is very difficult to conduct because of the heavy physical activity it requires for exposing the diseased roots from the soil, removal of the diseased regions from the root and supplying large volumes of the chemical solution, for which there are some registered chemicals. Thus, a simple and effective control method is still needed. It is very important to develop a simple and convenient inoculation method that is essential for conducting the studies about this particular plant disease.

In the case of white root rot disease, some inocula include naturally or artificially infested roots and twigs of host plants, incubated mycelia and their spores, and the most stable and reliable inoculum is the infested soil prepared by mixing the soil with wheat bran medium infested with *R. necatrix* (Araki, 1967). But the incubation period needed is long (20 days or more), keeping the uniformity in mycelium density in the medium is difficult and the mixing procedure is relatively hard to conduct especially in case of large scale field trials. The roots of test plants may be damaged by the wheat bran itself because of its high nutrient content, even if no pathogen is inoculated. Nakamura et al. (2007) used artificially infected small (ca. 50mm in length and ca. 10mm in diameter) Japanese pear twigs as an inoculum for *R. necatrix* and succeeded to induce the disease effectively. However, the fresh Japanese pear twig cannot be available at any time or everywhere and the size of the inoculum is not so small. We tried to develop a simpler and more convenient method to prepare the inoculum of *R. necatrix* and found the new inoculation method using the infested wooden chips made of disposable chopsticks.

MATERIALS AND METHODS

Fungal Isolates

Two fungal isolates of *R. necatrix* W563 was supplied by National Institute of Fruit Tree Science, Tsukuba, Japan were used for inoculation tests. W563 was isolated from diseased Japanese pear (*Pyrus pyrifolia*) root in Hiroshima prefecture, Japan and its severe pathogenicity on Japanese pear was confirmed (Kanematsu et al. 1997; Nakamura et al. 2007). The isolates were maintained on potato dextrose agar medium (PDA) at 25°C before the experiments. One year old seedlings of apple (*Malus x domestica*), Chinese crab apple (*Malus prunifolia*) and Japanese pear were grown in the small plastic pots filled with sterilized soil, and kept in an air conditioned green house at 25°C and used for the inoculation tests.

Procedures for inoculation

In the first experiment, disposable chopsticks (*Waribashi*) made of white birch (*Betula platyphylla*) wood were cut into small wooden chips (ca. 30 x 5 x 5mm) and autoclaved at 120°C for 20 minutes. A dozen of wooden chips were placed not to overlap each other on the mycelium of W563 colony incubated on PDA in Petri dish for 1 week at 25°C. Incubated at 25°C for 1 week, the W563 mycelium grew over the wooden chips adequately. The infected wooden chips were cut into uniform length (ca. 20mm), and attached onto the stem base of one year old Chinese crab apple seedling grown in the small plastic pot filled with sterilized soil and tightly fixed with Parafilm tape (chip attaching method). PDA medium strip (ca. 20 x 5 x 5mm) with the W563 mycelium incubated for 2 weeks at 25°C was also used for the inoculum as a control. PDA strip bearing the W563 mycelium was attached and fixed on the stem base of the seedling with Parafilm tape (PDA strip placing method). The inoculated regions were covered with the soil, then the seedlings

were kept in an air-conditioned green house at 25°C. The occurrence of disease symptoms was observed every day for six weeks. Five plants were used for each inoculation. As negative controls, autoclaved and non-inoculated wooden chips and PDA strips without mycelium were used.

In the second experiment, the plants used for the pathogenicity test were Chinese crab apple, Japanese pear and yellow lupine (*Lupinus luteus*). These plants, known as the host plants of white root rot fungus, were grown in small plastic pots filled with sterilized soil and kept in an air-conditioned greenhouse at 25°C. W563 were incubated on PDA plate at 25°C for more than 10 days. A dozen sterilized small wooden chips (ca. 10 x 5 x 5 mm) made of disposable chopsticks were randomly placed directly on the mycelium of W563 colony and kept at 25°C. After incubation for 2 weeks, the infected wooden chips were fully inserted perpendicularly into the soil adhering to the stem base of the potted seedlings to inoculate the fungus (chip inserting method). The autoclaved wooden chips that were not infected with W563 were inserted into the soil adhering to the stem base of the seedlings as controls. Five plants were used in each experiment.

After the inoculation test, the roots of inoculated plants were dug up and washed with tap water. The small part of the roots near the stem base was taken and its surface sterilized using 70% ethanol and 2% sodium hypochlorite solution. After washing with sterilized water twice, the root tissues were incubated on PDA at 25°C to observe the fungal growth.

RESULTS

In case of the chip attaching method, 4 of 5 Chinese crab apple seedlings inoculated with the white root rot fungus *R. necatrix* W563 showed severe symptoms whereas only 1 of 5 had symptoms, by means of PDA strip placing method (Table 1). For the diseased seedlings, the water soaked regions were seen along with the veins of the leaves by 3 weeks after the inoculation. The diseased plant showed decline in the newly developed twigs and wilting and drying of the leaves could be seen as the time passed. Leaf falling occurred followed by total decay of the seedlings by 6 weeks after the inoculation. The surface of the main roots and the whole of the fine roots were almost rotten on such decaying seedlings. Very severe and typical symptom occurred on the diseased Chinese crab apple seedlings inoculated with the pathogenic isolate in spite of the difference in the inoculation method by 6 weeks after the inoculation. One of 5 plants inoculated with W563 by chip attaching method did not showed the symptoms by 6 weeks after the inoculation and 4 of 5 plants inoculated with W563 by agar strip placing method either. The negative controls showed no symptom.

Table 1. The reactions of Chinese crab apple (*Malus prunifolia*) inoculated with *Rosellinia necatrix* W563 by infested wooden chip and mycelium bearing PDA strip inoculation methods ^{a)}

Fungal isolate inoculated	Inoculation methods	Number of plants	
		Diseased	Tested
<i>R. necatrix</i> W563	Infested wooden chip	4	5
	Mycelium bearing PDA strip	1	5
Control	Autoclaved wooden chip	0	5
	PDA strip	0	5

a) The results were observed by 6 weeks after inoculation.

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In the second experiment, W563 caused wilt of leaves and twigs by 3 weeks after the inoculation followed by leaf fall and total seedling decay with severe root rot by 6 weeks after the inoculation in all the inoculated plants without exception. The control plants that were not inoculated with W563 did not show any damage (Table 2).

The fungal isolates having arrow head shaped hyphae peculiar in *R. necatrix* could be isolated from the diseased seedlings but not from the healthy ones.

Table 2. The reactions of several plants inoculated with *Rosellinia necatrix* W563 by infested wooden chip inoculation method ^{a)}

Inoculated plants	Number of diseased / tested plant	
	W563	Control
Yellow lupine (<i>Lupinus luteus</i>)	5/5	0/5
Chinese crab apple (<i>Malus prunifolia</i>)	5/5	0/5
Japanese pear (<i>Pyrus pyrifolia</i>)	5/5	0/5

^{a)} The results were observed by 6 weeks after inoculation.

DISCUSSION

In case of the disease caused by *Fusarium* spp. and *Verticillium* spp., the suspension of conidia and/or pure cultured mycelium could be available in the inoculation test as well as the infested soil prepared by mixing sterilized soil with the pathogen (Iijima, 1983; Ogawa and Komada, 1984). Those fungi formed relatively much amount of spores in the medium but *R. necatrix* formed few. So in case of the inoculation of *R. necatrix*, the infested soil was mainly used as an inoculum. Among the inocula tested including the suspension of spores and/or mycelia, naturally or artificially infested root or twig of host plant, infested soil was the most stable and reliable material for the inoculation test (Araki, 1960). Although the preparation measure of wheat bran medium is easy and technique used in the incubation is simple, the terms for fully incubation enough to use for the inoculum is relatively long (20 days or more) and uniform incubation of the mycelium in whole of the medium was not always succeeded. As the fully incubated wheat bran medium is packed tightly, mixing work is very heavy especially in case of large scale experiment (Araki, 1967).

To reduce the heaviness and simplify the procedure of the work as well as to shorten the incubation period, the naturally infected roots and/or the artificially infected host plant twigs embedded in the soil were used for the inocula (Araki, 1967; Nakamura et al, 2007). However the naturally infected roots might be infested by the strain other than the designated *R. necatrix* isolate and/or utterly different microorganisms. Nakamura et al. (2007) used the artificially infested fresh Japanese pear twig for the inoculum. The twig was cut into fragments (ca. 50mm in length and ca. 10mm in diameter), inoculated with *R. necatrix* and embedded in the soil to inoculate on Japanese pear seedling in plastic pot. However, the fresh Japanese pear twig cannot be available at any time or everywhere in spite of their successful results.

We tried to use the infected wooden chips made of disposable chopsticks as an inoculum of *R. necatrix*. The disposable chopsticks made of white birch wood are so popular and very cheap in Japan that the material is very easy to obtain all the year round. Then we tried to reduce the size of inoculum and simplify the inoculum preparation procedures and the inoculation techniques. The size (ca. 10 x 5 x 5mm) is the smallest among the previous reports (Araki, 1967; Nakamura et al, 2007). The procedure for inoculum

preparation is completed only in the petri dish so that there may be low probability of the contamination by other microorganisms. It is very easy to prepare many inocula because only one chip is required for each seedling tested. There is no particular technique required but only inserting the chip perpendicularly into the soil adhering to the stem base of the seedling to be tested.

The inoculation results were highly successful in both chip attaching and chip inserting methods. In particular, all the inoculated plants showed very severe symptom by 6 weeks by the chip inserting method. The stability and the reliability of the inoculation by the chip inserting method were confirmed, at least in the pot level experiment. The inoculation failed only once in the chip attaching method. The causes for false results might be low pathogen density from insufficient incubation, poor contact between the seedling and the attaching inoculum by the contamination of unknown inhibitive material and/or insufficient oxygen supply caused by sealing with Parafilm tape. Regarding the low pathogen density, the problem was thought to be solved by extending the incubation period from 1 week to 2 weeks in the chip inserting method where all the inoculated plants showed severe symptoms. In addition, insufficient oxygen supply cannot occur in the case of chip inserting method. All re-isolated fungi are thought to belong to *R. necatrix* because of the existence of their arrow-head shaped hyphae that are peculiar for *R. necatrix*.

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